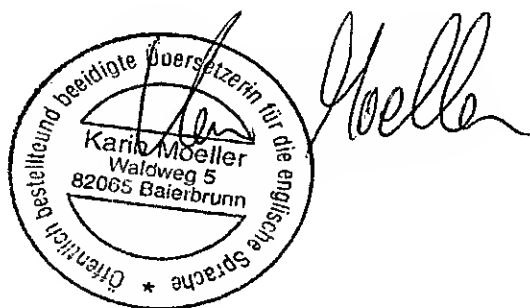I, Karin Moeller of Waldweg 5, D-82065 Baierbrunn, hereby certify that I am a sworn-in translator and that I am fully conversant with both the English and German languages and that the attached document is a true and complete translation of a public document, and that nothing has been added to or omitted therefrom.

München, this 19th day of May 2006

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Method of cloning a large viral genome

The present invention relates to a method of cloning large viral DNA genomes. The cloned viral genomes are infectious, i.e. that viruses (or virus particles) can infectiously be recovered from the cloned DNA by using suitable methods. The invention further relates to an artificial circular chromosome that can be obtained by means of such a method as well as to its use, and to a method of mutagenesis of large viral genomes by using the artificial circular chromosome.

A number or large viral genomes exists, which are so large that it is difficult to clone them, to analyze them and to carry out an aimed mutagenesis. This is amongst others due to the fact that the formerly used methods need the use of multiple recombination steps in eucaryotic cells, which firstly are rare and secondly error-prone and thirdly they are practically uncontrollable. This requires complex selection methods to be able to isolate and characterize desired mutants.

Various viruses with a large DNA are important pathogens, either for human beings or for animals or plants, partly causing severe or even fatal diseases. Reference is made by way of example to the cytomegalovirus (CMV) which is counted among the most important human pathogenic viruses. CMV has a high prevalence, which causes severe diseases, especially in immunologically immature patients or in immunocompromised patients (1). Since human cytomegalovirus (CMV) and mouse cytomegalovirus (MCMV) show several similarities in their biology and pathogenesis (2), infection of mice with MCMV has become an intensively examined <u>in vivo</u> model to study the pathogenesis of CMV infection. The 235 kb large genomes of both human and mice CMV are the largest genomes of mammalian DNA viruses. Sequence analysis of the human and mice CMV genomes reveal a similar genetic organization and a coding capacity for presumably more than 220 polypeptides (3, 4, 5).

Information on the function of a majority of CMV gene products is still rather limited because of the size of the viral DNA and the associated difficult manipulatability of the viral genome. This is e.g. in contrast to the situation regarding the Herpes simplex virus (α-Herpes viruses) (6), which has been studied in detail and for which the function of many viral genes could be elucidated. Thus, there is a need for CMV clones and CMV
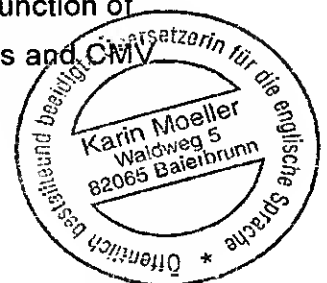
mutants, since they have been difficult to obtain because of their large DNA and their slow replication kinetics.

Thus, there is also a need for cloning methods for other large viral genomes, particularly Herpes virus genomes, which revealed the same problems, as the ones exemplarily described for CMV.

A method for insertional mutagenesis has been developed for the disruption and deletion of CMV genes (7, 8). However, since the frequency of homologous recombinations in eukarytoic cells is low, the method is quite ineffective. In addition, adventitious deletions and the production of undesired recombinant viruses have frequently been observed (7, 9). Although selection procedures have improved the original method (9, 10, 11), generation of CMV mutants remains a laborious and often unsuccessful task. Recently, a method for constructing recombinant Herpes viruses from cloned overlapping fragments (12) has been applied to CMV (13). This is a major improvement in comparison with the above-described methods in that said method generates only recombinant viruses and obviates selection against non-recombinant wild-type viruses. However, the resultant mutants are still the product of several recombination events in eukaryotic cells that are difficult to control. Correct reconstitution of the viral genome can only be verified after growth and isolation of the viral mutant.

Thus, there was still need of providing a method for cloning large, complete but in any case replicable (so-called "infectious") viral genomes, which requires as few recombination steps as possible in eukaryotic cells and in which therefore an isolation and selection of the recombinants and their selection is dispensable.

This object is solved by the method cited in claim 1. Advantageous embodiments are defined in the dependent claims.

This object is solved according to the present invention in that a method for cloning large viral genomes is provided, comprising the following steps:

    a) introducing a sequence (1) containing sequences of a cloning vehicle, into a cell containing the viral genome to be cloned, and

b) recombining the sequence (1) of step a) with the DNA fragment to be cloned preferably via homologous recombination so that an artificial circular chromosome is obtained.

The homologous recombination can for instance be caused in that the sequence (1) contains portions that are homologous to the DNA to be cloned.

The method is particularly suited for those viral genome that are larger than 100 kb (preferably larger than 200 kb). The method is particularly interesting for cloning large viral DNA, such as the DNA of Herpes viruses, wherein particularly the human cytomegalovirus and the cytomegalovirus in mouse shall be mentioned. These viruses are important pathogens for the human being and their cloning and subsequent mutagenesis is made possible only by the method according to the invention. Further suitable viral genomes are those of other DNA viruses or all Herpes virus genomes (Herpes simplex virus type 1 and 2, the Epstein-Barr virus, the Varicella-Zoster virus, the human Herpes viruses 6, 7 or 8 (HHV6, HHV7, HHV8), animal Herpes viruses, such as Pseudorabies virus, or bovine Herpes viruses 1, 2, 3, 4.

Preferably, eukaryotic cells, preferably mammalian cells, insect cells or yeast cells, are used as host cells for step a); primary fibroblasts of humans (e.g. human foreskin fibroblasts (HFF)) and of mouse (for example fibroblasts of (BALB/c)-mice) or NIH3T3 fibroblasts (ATCC CRL1658) can here in particular be used.

The introduction of sequence (1) into eukaryotic cells is carried out by a calcium precipitation method, an electroporation method or a lipofection method or by means of methods of the latest prior art. The sequence (1) of step a) is preferably loxP-flanked or flanked by unique restriction sites, which enables its later resection by the recombinase Cre or restriction enzymes. The virus DNA to be cloned may possibly also be introduced into a suitable cell first, if the cell containing same proves to be unsuitable for cloning.

In a further step, the chromosome obtained is introduced according to the above-mentioned method into bacteria such as E. coli. This introduction may for instance be carried out by electroporation or any other method known in the prior art. The cloning vehicle is preferably a plasmid, e.g. the plasmid pRP2 or pRP3, as described below. Any vehicle is suitable, which together with the DNA to be cloned may form an artificial ring

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chromosome, such as a bacterial chromosome (BAC). Suitable cloning vehicles are low-copy vectors, since the stability of the cloned DNA is only ensured by the low number of copies of the plasmids. Further suitable vehicles are derivatives that are derived from the known mini-F-plasmids of E. coli. The mini-F-plasmid contains the bacterial genes (functions) repE (for the replication), parA, B, C (for the distribution of the plasmid on daughter bacteria and the strict supervision of the number of copies) ovi_s (origin of replication; for the replication).

The invention further refers to an artificial circular chromosome, which can be obtained by one or several of the methods described in claims 1 to 14. The artificial circular chromosome is preferably bacterial and contains the entire DNA sequence of a virus that is essential for the reproduction thereof, as well as sequence (1) of step a) of claim 1.

In an especially preferred embodiment, the artificial circular chromosome contains the DNA sequence of Herpes viruses, especially preferred the DNA sequence of the human cytomegalovirus, or the DNA sequence of the cytomegalovirus of mouse and an antibiotic resistance gene.

The invention further refers to the use of an artificial circular chromosome, which can be obtained by the above-mentioned methods, for the mutagenesis of large viral DNA genomes, particularly the insertion of therapeutically important genes and the production of vectors for the somatic gene therapy.

The invention also refers to a method of mutagenesis of large viral genomes, comprising the following steps:

A) cloning a large viral genome according to one or several of claims 1 to 14;

B) introducing the obtained artificial chromosome into a bacterial host cell or into yeast; and

C) homologous recombination of the chromosome in bacteria or in yeast by a mutant allele.

The present invention also refers to cells that include an artificial circular chromosome of the above-mentioned type.

The artificial circular chromosomes are also suitable for a gene-therapeutic use or as part of a vaccine.

The Figures describe the present invention in detail.

Figure 1 shows a strategy for the cloning and mutagenesis of a large viral genome.

(A) A large viral genome and a recombination plasmid with bacterial vector sequences were introduced into eukaryotic cells to generate a recombinant DNA (BAC). This can be performed by various methods, e.g. infection, transfection, electroporation, etc. Circular DNA was isolated from the infected cells and transferred into E. coli. (B) Mutagenesis of the BAC was performed in E. coli by homologous recombination with a mutant allele, and the mutated BAC was transfected into eukaryotic cells to reconstitute recombinant viral genomes or viruses (C).

Figure 2 shows the construction and structure of the MCMV bacterial artificial chromosomes (BACs) pSM3 (a) and pSM4 (b) and the structure of the derived recombinants MC96.73 and MC96.74. The EcoRI restriction map of the right-terminal end of the genome of the MCMV strain Smith is shown above. The recombinant plasmids pRP2 and pRP3 contain 2.2 and 6.6 kb of flanking MCMV homologous sequences (white boxes), the BAC vector (grey) and the gpt gene (hatched), flanked by loxP sites (black). The EcoRI restriction map of the BACs pSM3 and pSM4 is shown thereunder. The terminal EcoRI fragments of the MCMV genome are fused into the BAC plasmids pSM3 and pSM4, resulting in new fragments of 22.9 and 24.3 kb, respectively. The linear genomes of the recombinant viruses MC96.73 and MC96.74 contain terminal EcoRI fragments of a length of 12.3 and 13.7 kb, respectively. Additional restriction enzyme sites are indicated by BamHI (B), HindIII (H) and SfiI (S).

Figure 3 shows the structural analysis of the bacterial artificial chromosomes (BACs) pSM3 and pSM4 (a) and the reconstituted viral genomes (b, c). (a) Ethidium bromide-stained agarose gel of EcoRI-digested BACs isolated from E. coli cultures (lanes pSM3 and pSM4) and of wild-type MCMV DNA isolated from purified virions (lane wt). (b)
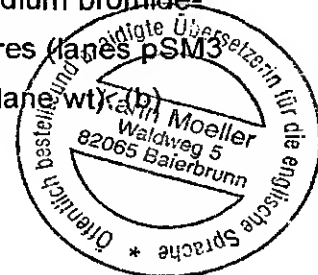
Restriction enzyme analysis of the viral MC96.73 and MC96.74 genomes. The BACs pSM3 and pSM4 were transfected into mouse embryonic fibroblasts and the supernatant of the transfected cells was used for infecting new cells. The DNA isolated from the infected cells and wild-type MCMV DNA were digested with EcoRI and separated by electrophoresis on 0.6% agarose gels for 14 hours. The EcoRI O(O) and the vector fragments (v) are indicated and the size of the additional bands is shown at the left side. (c) Separation of the EcoRI fragments shown in (b) after electrophoresis for 28 hours.

Figure 4 shows the construction of the MCMV ie1 mutant MM96.01 (a), the structural analysis of the mutated bacterial artificial chromosome (BAC) (b) and the genome of the ie1 mutant MM96.01 (c). The HindIII restriction between the HindIII K and L fragments of the genome of the MCMV strain Smith (top) was removed by mutagenesis using the EcoRI/HpaI fragment (hatched region). The exon-intron structure of the ie1 and ie3 genes is indicated, and the protein-coding sequences are depicted as hatched boxes. The mutation results in a frame shift after 273 codons and in the formation of a new stop codon after another 9 codons (black box). The open box denotes the part of the ie1 open reading frame which is not translated in the mutated virus. (b) Ethidium bromide-stained agarose gel of the HindIII-digested parental BAC pSM3 and of the mutated BAC pSMie1. (c) HindIII pattern of the genomes of recombinant virus MC96.73 and of the ie1-mutant MM96.01. The HindIII K and L fragments and the new 15.2-kb fragment are indicated at the left side and the size of some HindIII fragments is shown at the right margin.

Figure 5 shows the absence of pp89 in cells infected with the ie1 mutant MM96.01. Mouse embryonic fibroblasts (MEF) were either not infected, infected with the recombinant virus MC96.73 or with the ie1 mutant MM96.01 in the presence of cycloheximide (50 μg/ml) for 3 hours for achieving a selective expression of the immediate-early proteins (16). After removal of cycloheximide, actinomycin D (5 μg/ml) was added and the proteins were labeled with ^{35}S-methionine (1200 ci/mmol) for 3 additional hours. Lysis of cells and immunoprecipitations were performed as described (28) using antiserum 3/1 directed to the C-terminus of the ie1 protein pp89 (a) and the ie1/ie3-specific antipeptide serum b5-1 (b) (16, 28). A long exposure of the autoradiograph (b) is shown in (c).

The invention will now be described in detail in view of the examples and the Figures.
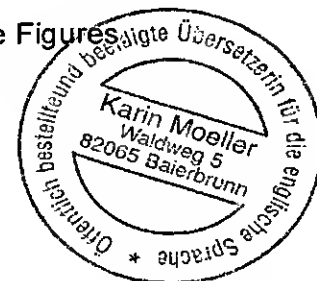
Example 1: Generation of Viruses and Cells

The propagation of MCMV (strain Smith, ATCC VR-194, ATCC, Rockville, Md.) in BALB/c mouse embryonic fibroblasts (MEF) and NIH3T3 fibroblasts (ATCC CRL1658) has been described earlier (14, 15). Recombinant viruses were generated according to published protocols (8, 9, 15). To generate virus progeny from bacterial artificial chromosomes (BACs), BACs (about 0.5-1 µg) were transfected into MEF by employing the calcium phosphate precipitation technique as described in (15, 16).

Example 2: Isolation of viral DNA and BACs

Plasmid cloning is carried out according to standard techniques (20). Restriction enzymes were purchased from New England Biolabs (Bevety, MA). Wild-type MCMV DNA was generated from virions, and total cell DNA was isolated from infected cells, as described earlier (14, 17). Circular viral DNA was isolated by the method of Hirt (18) and electroporated into electrocompetent E. coli DH10B strains (19). BACs were isolated from E. coli cultures using an alkaline lysis procedure (20) and purified by precipitation with polyethylene glycol (20).

Example 3: Plasmids and Mutagenesis

For construction of recombination plasmids pRP2 and pRP3, a 17 kb HindIII/BamHI subfragment of the MCMV HindIII E' fragment (17) was subcloned into pACYC177 (21). The EcoRI fragments O, b, f, and g within the HindIII E' fragment (14, see Fig. 2) were deleted and an EcoRI/NotI adapter was added to create the plasmid pHE'ΔE. The E. coli guanine phosphoribosyl transferase (gpt) gene controlled by the thymidine kinase promoter of the Herpes-simplex virus and followed by the early polyadenylation site of the SV40 early gene was flanked at one side by a tandem loxP site and then cloned into the plasmid pKSO, a derivative of the BAC vector pBAC108L (19) with modified polylinker (PmeI-NsiI-PacI-BamHI-PmeI-AscI), after it was first of all inserted into a BamHI site that was located between tandem-loxP sites and was previously cloned in pBluescript KS+ (stratagene). The pKSO-gpt plasmid was linearized by NotI between the two loxP sites and inserted into the only NotI site of the plasmid pHE'ΔE. pRP2 and pRP3 differ from each other in the orientation of the pKSO-gpt sequence (Fig. 2).
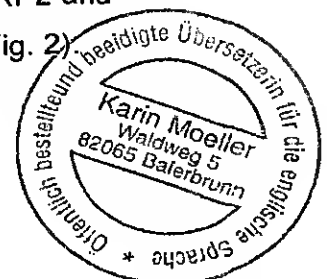
To construct the mutagenesis plasmid pMieFS, a 7.2 kb HpaI/EcoRI fragment (Fig. 4a) from plasmid pIE111 (23) was inserted into plasmid pMB096 (24), and the HindIII site in the insert was filled and destroyed by treatment with Klenow polymerase. Mutagenesis of the MCMV BAC was performed by homologous recombination in the E. coli strain CBTS (25) following published protocols (24, 25).

Example 4: Strategy for cloning and mutagenesis of the MCMV Genome

The known methods for manipulating CMV genomes and other large viral genomes are only applicable and successful to a limited degree because they are based on homologous recombination in eukaryotic cells. To make the MCMV genome and other large virus genomes more accessible to mutagenesis, infectious bacterial artificial chromosomes (BAC) of MCMV were generated in E. coli. Since Herpesviruses circularize their genome after cell entry (6, 26), and the plasmid-like circular intermediates occur early in the Herpes viral replication cycle (27), the strategy depicted in Fig. 1a was adopted for cloning the MCMV genome. In a first step a recombinant virus was produced that contained a bacterial vector integrated into its genome. After selection of recombinant viruses using the selection marker guanine phosphoribosyl transferase (gpt) (9), circular intermediates accumulate in infected cells. After isolation and electroporation of the circular intermediates into E. coli, the CMV-BAC is available to all gene technology techniques using E. coli. Transfection of the mutated BACs into eukaryotic cells should finally reconstitute viral mutants (Fig. 1).

Example 5: Generation of recombinant Viruses and BACs

We have shown previously that a large region at the right-terminal end of the MCMV genome is not essential for replication in vitro (18). Therefore, this region was chosen for integration of the BAC vector and the selection marker gpt (Fig. 2a, b). To find out whether integration of the BAC vector into the viral genome could be achieved in both orientations, two different recombination plasmids pRP2 and pRP3 were produced (Fig. 2a, b). For generation of BAC-containing viral genomes the recombinant virus ΔMC95.21 was used that has a lacZ insertion in the EcoRI-O fragment of its genome. This allowed the identification of integration events by screening for white plaques after staining with 5-bromo-3-chloro-indolyl-β-galactopyranoside (X-Gal). Recombinant viruses with
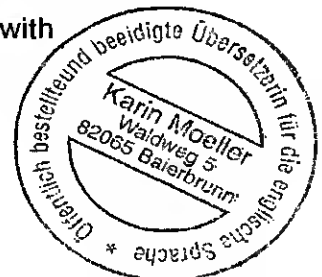
integrated vector sequences were enriched using the gpt marker. Finally, circular viral DNA was isolated from infected cells and electroporated into E. coli.

A high percentage of bacterial clones (about 80%) contained the expected complete plasmids. In comparison with DNA isolated from MCMV virions, the BACs pSM3 and pSM4 contained additional EcoRI fragments having a length of 22.9 and 24.3 kb, respectively (Fig. 3 a), depending on the orientation of the integrated vector (Fig. 2a, b). The additional bands resulted from the fusion of the terminal EcoRI fragments, indicating the circular nature of the BACs (Fig. 2); as expected, the 5.7 kb EcoRI-O fragment was missing in the BACs (see Figs. 2 and 3a), and the vector sequences resulted in a double band at 6.4 kb (designated as v in Fig. 3a). In the BAC pSM3 the 2.5 kb EcoRI Z fragment was enlarged by 1.4 kb of the gpt and vector sequences, leading to a 3.85 kb fragment (Fig. 3, lane pSM3). Southern blot analysis and characterization of the BACs with restriction enzymes HindIII, XbaI and BamHI (data not shown) confirmed the successful cloning of the total genome of these MCMV recombinants into E. coli.

Example 6: Reconstitution of virus progeny from MCMV- BACs

Transfection of the BACs pSM3 and pSM4 into mouse embryonic fibroblasts led to the development of plaques. New cells were infected with the supernatant from cells transfected with pSM3 and pSM4. Total DNA was isolated when cells showed a complete cytopathic effect. EcoRI cleavage of the isolated DNA resulted in a similar pattern as cleavage of the BACs pSM3 and pSM4 (cf. lanes MC96.73 and MC96.74 in Fig. 3b and lanes pSM3 and pSM4 in Fig. 3a). DNA isolated from infected cells comprises circular, concatemeric and linear viral DNA, which is already packaged into capsids. Therefore, the amount of the 22.9 kb and 24.3 kb fragments resulting from the fusion of the terminal EcoRI fragments was submolar (Fig. 3b, lanes MC96.73 and MC96.74). Furthermore, the terminal EcoRI fragments deriving from linear genomes reappeared. The terminal EcoRI fragment (12.3 kb) was discovered in the genome of the recombinant MC96.73 and also in wild-type MCMV (Fig. 3c, lanes MC96.73 and wt). In the recombinant MV96.74 the terminal EcoRI fragment F was enlarged by a 1.4 kb vector sequence (see Fig. 2b), resulting in a double band at 13.7 kb (Fig. 3c, lane MC96.74). Digestion with the restriction enzymes BamHI and XbaI produced all expected bands (data not shown). Thus, it was possible to generate CMV recombinants from one large plasmid which had not been manipulated prior to transfection.
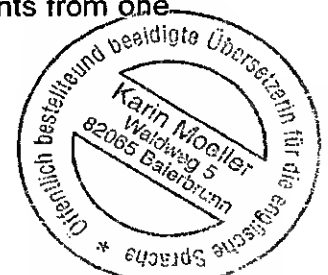
Example 7: Construction of an MCMV ie1 mutant by homologous recombination in E. coli

To test the efficacy of targeted mutagenesis in E. coli, a small mutation was introduced into the (immediate-early) (ie) region of the MCMV genome. At least two alternative spliced transcripts arise from the ie region (Fig. 4a) that encode the 89 kDa ie1 protein and the 88 kDA ie3 protein (16). Due to the complex structure of the ie1/ie3 transcription unit, disruption of the ie1 gene is probably difficult to achieve by conventional recombination techniques without affecting the expression of the ie3 gene. Therefore it was not known whether the MCMV ie1 protein is essential for virus replication. To disrupt the ie1 reading frame (595 codons) a reading frame mutation was introduced at a HindIII site in exon 4 of the ie1 gene. The mutation caused the original reading frame to finish after codon 273 and created a new stop codon after 9 additional codons (Fig. 4a). The mutation was constructed on a 7.4 kb EcoRI/PhaI fragment (Fig. 1a) and subsequently transferred to the BAC pSM3 by homologous recombination in E. coli employing a two-step replacement strategy (24, 25). The mutagenesis procedure resulted in the loss of the HindIII K and L fragments and the generation of a new 15.2 kb fragment (Fig. 4b). The EcoRI and BamHI patterns of the BACs were unchanged (data not shown), confirming that the BACs remained stable during the mutagenesis procedure. Transfection of the mutated BACs pSM4-ie into MEF led to plaque formation. Total DNA was isolated from infected cells and analyzed by HindIII digestion. As expected, the HindIII K and L fragments were replaced by the 15.2 kb fragment in the genome of the ie1 mutant virus MM96.01 (Fig. 4c). Obviously, the mutation introduced into the MCMV BAC was maintained after reconstitution of the mutant virus.

Absence of the ie1 protein in infected MM96.01 cells was confirmed by immunoprecipitation. An antiserum directed to the carboxy terminus of the ie1 protein detected the ie1 protein in lysates of MC96.73-infected cells, but did not precipitate any protein in lysates of MM96.01-infected cells (Fig. 5a). An ie1/ie3-specific antiserum, B5/1, (16, 28) detected 2 proteins of 89 and 88 kDa in lysates of MC96.73-infected cells and one protein of 88 kDa in lysates of MM96.01-infected cells (Fig. 5b). In the MM96.01 lane the 89 kDA ie1 protein was clearly missing and only the 88 kDA ie3 protein was detected (Fig. 5b). A longer exposure of the autoradiograph revealed a 36 kDA protein in MM96.01-infected cells (Fig. 5c). The apparent molecular weight of this polypeptide is in
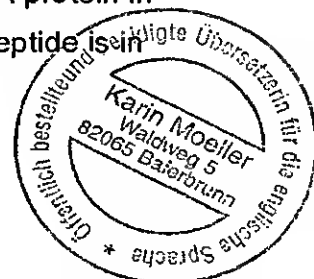
agreement with the expected molecular mass for the truncated ie1 protein and with the migration behavior of various mutated ie1 proteins (28).

Thus, we drew the conclusion that the ie1 protein pp89 is not necessary for replication of the ie mutant MM96.01.

Example 8: Methods of Mutagenesis

Following the published protocols of O'Connor et al. (24) and Kempkes et al. (25), mutagenesis of the MCMV BACs was performed by homologous recombination in the E. coli strain CBTS which carries a recA allele and a temperature-sensitive suppressor (25). The BAC pSM3 and the shuttle plasmid pMieFS were successively electroporated into E. coli CBTS, and clones which contained cointegrates were selected at 42°C on agar plates with chloramphenicol (12.5 µg/ml) and tetracyclin (10 µg/ml). The cointegrates were analyzed for the desired structures by restriction enzyme analysis. The separation of the cointegrates was made possible by incubation of the bacterial clones at 30°C on agar plates only with chloramphenicol. The plasmids were identified by screening for tetracyclin-sensitive clones and analyzed by HindIII cleavage to determine whether a mutation had been achieved or whether there had been a return to the parental sequence.

Example 9: Construction of recombinant MCMV

Recombinant viruses were generated either by cotransfection of viral DNA and linearized recombination plasmid, as described above (8, 15), or by electroporation of the recombination plasmid into NIH3T3 cells using the BioRad gene pulser (250 V, 960 µF), followed by a superinfection with MCMV 8 hours later. Recombinant viruses were selected with mycophenolic acid and xanthine in accordance with published protocols (9).

Example 10: Isolation of viral DNA

Wild-type MCMV DNA was prepared from virions and purified by CsCl gradient centrifugation as described previously (14). For a characterization of the reconstituted genomes, the whole cell DNA was isolated from infected cells. The cells were harvested
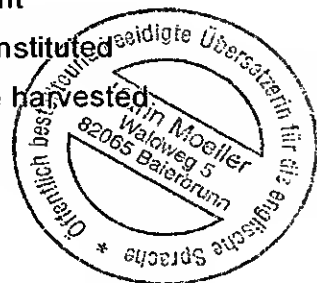
by trypsination, followed by centrifugation at 800 g for 5 minutes, and lysed in 50 mM Tris-HCl, pH 8, 20 mM EDTA, 0.5% SDS with 0.5 mg/ml proteinase K. After incubation at 56°C for 12 h the DNA was extracted twice with phenol/chloroform/isoamyl alcohol (50:48:2) and precipitated with ethanol. The DNA fragments were separated by electrophoresis on a 0.6% agarose gel, as described previously (14).

Circular viral DNA was isolated by the method of Hirt (18). Infected cells from a 60-mm tissue culture dish were lysed in 1 ml of buffer A (0.6% SDS, 10 mM EDTA pH 7.5), and 0.66 ml of 5 M NaCl were added, followed by incubation at 4°C for 24 h. The samples were centrifuged at 15,000 g and 4°C for 30 min, the supernatant was extracted with phenol, and DNA was precipitated with ethanol. The DNA was again dissolved in 30 µl TE (10 mM Tris-HCl pH 8, 1 mM EDTA) and dialyzed against TE. 10 µl of the DNA were introduced by electroporation into electrocompetent E. coli DHI OB using a BioRad gene pulser (2.5 RV, 25 µF, 400 ohm). Transformants were selected on agar plates containing 12.5 µg/ml chloramphenicol.

Example 11: Isolation of BACs and reconstitution of recombinant viruses

BACs were isolated from 100 ml cultures which had been grown overnight at 37°C in the presence of 12.5 µg/ml of chloramphenicol, using the alkaline lysing technique (20). The plasmid DNA was further purified by precipitation with polyethylene glycol (20). One tenth of the isolated plasmids (about 0.5 to 1 µg) was used for analysis by restriction enzyme cleavage or for reconstitution of the virus progeny by caicium phosphate transfection in MEF.

Example 12: Metabolic labelling and Immunoprecipitation

A selective expression of wt or mutated MCMV immediate-early proteins was achieved by infection of MEF in the presence of cycloheximide (50 µg/ml) which 3 hours later was replaced by actinomycin D (5 µg/ml). Cells were then labelled with [35S]-methionine (1200 Ci/mmol; Amersham, Braunschweig, Germany) for another 3 hours. The cells were lysed and immunoprecipitations were performed using the antipeptide serum b5/1 (28, 16) and the antiserum 3/1 which is directed against the C-terminus of the ie1 protein pp89.
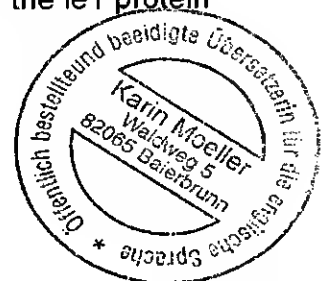
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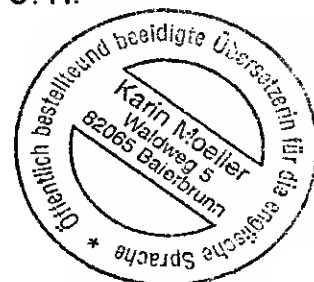
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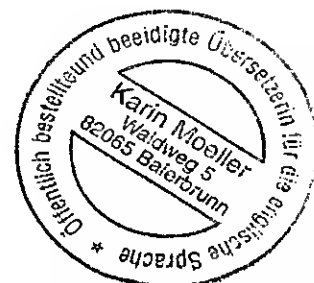
Patent Claims

1. A method for cloning a large viral genome, comprising the following steps:

   a) introducing a sequence (1) containing sequences of a cloning vehicle, into a cell containing the viral genome to be cloned, and

   b) recombining the sequence (1) of step a) with the viral genome to be cloned preferably via homologous recombination so that an artificial circular chromosome is obtained.

2. A method as claimed in claim 1, characterized in that the viral genome is larger than 100 kb (preferably larger than 200 kb).

3. A method as claimed in claim 1 or 2, characterized in that the large viral genome originates from a DNA virus.

4. A method as claimed in claim 3, characterized in that the viral DNA originates from Herpes viruses.

5. A method as claimed in claim 4, characterized in that the Herpes virus DNA is the DNA of the human cytomegalovirus.

6. A method as claimed in claim 4, characterized in that the Herpes virus DNA is the DNA of the mouse cytomegalovirus.

7. A method as claimed in one or a plurality of the above-mentioned claims, characterized in that the cell that is used in step a) is an eukaryotic cell.

8. A method as claimed in claim 7, characterized in that the eukaryotic cells are mammalian cells, preferably primary fibroblasts, human foreskin fibroblasts (HFF) or mouse embryonic fibroblasts of BALB/c-mice.

9. A method as claimed in claim 7, characterized in that the eukaryotic cells are NIH 3T3 fibroblasts.
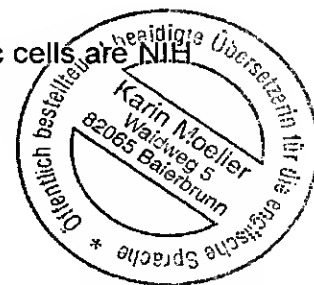
10. A method as claimed in one or several of the preceding claims, characterized in that sequence (1) is itransfected into the cells by a calcium phosphate precipitation method or an electroporation method.

11. A method as claimed in one or several of the preceding claims, characterized in that the sequence (1) of step a) is flanked by loxP sites or by restriction sites that do not appear in the residual genome.

12. A method as claimed in one or several of the preceding claims, characterized in that the chromosome obtained is introduced into E. coli in a further step c).

13. A method as claimed in claim 12, characterized in that the introduction into E. coli is implemented by electroporation.

14. A method as claimed in one or several of the preceding claims, characterized in that the cloning vehicle is a low-copy plasmid, preferably a mini-F-plasmid derivative.

15. An artificial circular chromosome, obtained by one or several of the methods described in claims 1 to 14.

16. An artificial circular chromosome as claimed in claim 15, characterized in that it is a bacterial chromosome (BAC).

17. An artificial circular chromosome as claimed in claim 15 or 16, characterized in that it contains the entire DNA sequence of a virus that is essential for the virus reproduction, as well as sequence (1) of step a).

18. An artificial circular chromosome as claimed in claim 17, characterized in that it contains the DNA sequence of a Herpes virus as well as sequence (1) of step a).

19. An artificial circular chromosome as claimed in claim 18, characterized in that it contains the DNA sequence of the human cytomegalovirus.
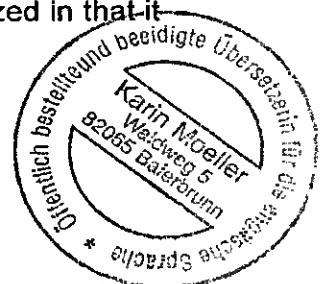
20. An artificial circular chromosome as claimed in claim 18, characterized in that it contains the DNA sequence of the mouse cytomegalovirus.

21. Use of an artificial circular chromosome according to one or several of claims 15 to 20 for a mutagenesis of large DNA fragments.

22. A method for mutagenesis or large viral genomes, comprising the following steps:

A) cloning a large viral genome according to one or several of claims 1 to 14;

B) introduction of the obtained chromosome into a bacterial host cell or yeast; and

C) homologous recombination of the chromosome with a mutant allele.

23) A cell, including an artificial circular chromosome as claimed in one of claims 15 to 20.

24) An artificial circular chromosome as claimed in one of claims 15 to 20 as a drug, preferably as a vaccine (somatic gene therapy).

25) Use of BAC plasmids for cloning large circular DNA viral genomes.
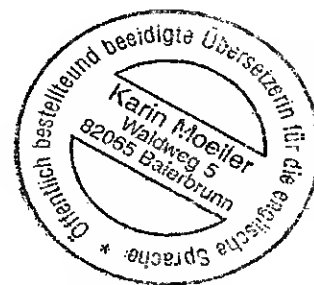
Abstract

A method of cloning a large viral genome

The present invention refers to a method of cloning a large viral genome. The invention also refers to an artificial circular chromosome that can be obtained by such a method and to its use, and a method for mutagenesis of large viral genomes by using the artificial circular chromosome.
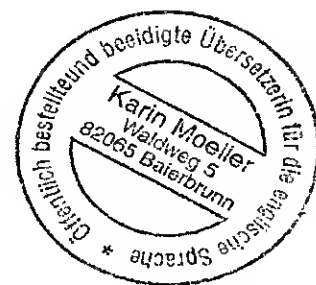
Drawings

Fig. 1:

Klonierungsvehikel: cloning vehicle

Genom: genome

Eukaryontische Zelle: eukaryotic cell

Homologe Rekombination: Homologous recombination

Cointegrat: cointegrate

Mutantes BAC: mutant BAV

Mutantes Genom: mutant genome

Fig. 5:

Nicht infiziert: not infected

N-Terminus: N-term